Biomechanical Forces Activate Tissue Transglutaminase Resulting in Vascular Remodeling and Stiffening

By
Sean Melucci

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ABSTRACT

Arterial compliance plays an important role in the proper functioning of the human cardiovascular system. Loss of large arterial compliance leads to increased isolated systolic hypertension and ventricular hypertrophy[1], which results in increased risk of major adverse cardiovascular events.[1,2] Arterial stiffening occurs as a result of vascular remodeling through extracellular matrix (ECM) cross-linking and smooth muscle cell proliferation. Current hypertension treatments are only able to treat classical essential hypertension with poor success rates in controlling systolic blood pressure.[3] There are no targeted treatments available to target the age-induced arterial stiffening due to outward vascular remodeling.

Tissue transglutaminase (TG2) is an important enzyme involved in arterial remodeling with a further emerging potential as a therapeutic target for aging induced isolated systolic hypertension. TG2 is a multifunctional enzyme with a main crosslinking functionality that produces unbreakable "isopeptide" bonds between ECM proteins leading to increased vascular stiffness.[4,5] While there is substantial evidence that TG2 drives pathobiology of vascular stiffening in aging, significant gaps remain in our understanding of the mechanisms of TG2 regulation and action in the vasculature. Here, we hypothesize that 1) augmented biomechanical strain is a central mechanism contributing to TG2 activation and 2) TG2 crosslinking function is critical to the vascular remodeling program. Lack of mouse models and specific inhibitors has hindered our ability to study TG2 crosslinking function in vascular biology and disease. Therefore, we generated a novel TG2-C277S mutant mouse model that expresses a crosslinking deficient TG2 protein, to selectively exclude crosslinking dependent mechanisms of TG2 action in the vasculature.

In this study, we found that human aortic endothelial cell secretion of TG2 to the ECM peaks at normal physiological stretching (10% strain) and decreases at pathophysiological

stretching (20% strain). In human aortic smooth muscle cells, TG2 secretion to the ECM and activation proportionally increase due to increased stretching strain. Extending our studies of vascular remodeling to our mouse model, we found reduced stiffness initially in the common carotid arteries of TG2 C277S and TG2 -/- mice. After hypertensive remodeling, we found that TG2 crosslinking is essential to hypertension induced vascular remodeling. We thus suggest that in aging isolated systolic hypertension (and increased pulse pressure) increases biomechanical strain, activating more TG2 in the tunica media, while decreasing its expression in the intima, leading to outward remodeling and vascular stiffening.

Advisors: Dr. Lakshmi Santhanam and Dr. Sharon Gerecht

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INTRODUCTION

Significance

Heart disease and stroke remain the leading causes of death worldwide, and in the United States specifically, they have been the leading causes of death since 1921.[8] Hypertension specifically has had a continuous increased death rate in the United States, with an estimated 31.1% of the world's population diagnosed with hypertension in 2010.[8] In 2015, an estimated 874 million adults had a systolic hypertension equal to or above 140 mmHg.[8] The main culprit of aging associated isolated systolic hypertension is arterial stiffening. Left unchecked, arterial stiffening increases the risk of a serious adverse cardiac event. [1,2] The gold standard treatment for isolated systolic hypertension is to use certain antihypertensive and diuretic drugs combined with lifestyle changes in both diet and exercise.[3,9] To this day, there are no drugs on the market able to treat the arterial stiffening behind isolated systolic hypertension. In order to design a specific therapeutic for isolated systolic hypertension due to aging that precisely targets large arterial remodeling, potential molecular targets must be characterized further.

Background

Arterial Structure and Compliance

Arteries are composed of a single endothelial cell layer (tunica intima), multiple layers of smooth muscle cells with an elastic lamina made up of primarily collagen and elastin (tunica

media), and a layer of connective adventitia made up of extracellular matrix (ECM) proteins such as collagen, elastin and fibronectin as diagramed below in **Figure 1**.

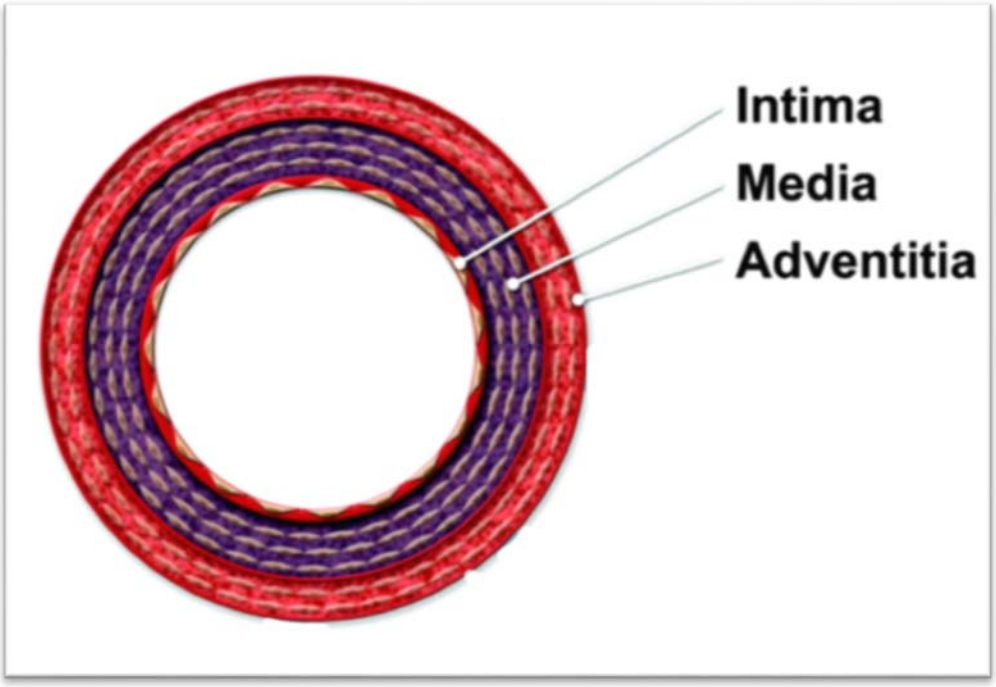

Figure 1: Arterial Structure.[10] Arteries are comprised of three layers: Tunica Intima (shown in deep red), Tunica Media (shown in purple), and Adventitia (shown in bright red/orange). (Permission obtained from publisher of citation for use of illustration).

The main function of arteries is to actively manipulate blood flow to supply oxygen and nutrients to specific tissues as necessary. Under short-term changes in blood flow, the smooth muscle cell layers within the tunica media contract or relax, which allow for immediate active changes in the artery diameter. In response to long term changes in blood flow and blood pressure, structural changes in the artery occur changing its compliance. Vessel compliance refers to the relationship between the lumen volume of the vessel and the pressure exerted within the lumen. The vessel compliance can also be measured and varies when the vessel in a relaxed state (passive compliance) or in a contracted state (active compliance).

While arteries must be able to manipulate blood flow to perfuse tissues in a timely manner (mostly controlled by smaller resistance arteries), large arteries specifically must be able to withstand the high pressures and hoop stress exerted directly after a period of contraction and relaxation of the ventricles. Therefore, the compliance of large arteries must have a perfect balance for the artery to function properly. If too compliant, the vessel is at risk of bursting and will not be able to keep blood flowing effectively. If not compliant enough, the vessel is not able to absorb the energy generated from the high pressure exerted during systole, which puts recoil stress back onto the heart that eventually leads to ventricular hypertrophy. The ability of large arteries to change their structure and modify their compliance is important for proper functioning and maintenance of structural integrity over a wide range of operating conditions of hoop stress. However, under pathophysiological conditions involved in chronic isolated systolic hypertension, the resulting changes can exacerbate the problem.

Hypertension and Vascular Remodeling

Under chronic hypertensive conditions, arteries are able to adapt their structure permanently to withstand the systemic blood flow increase. The vessel's response, known as vascular remodeling, leads to increased stiffness.[11] This can occur in three different ways affecting all three zones of the arterial structure including intimal hyperplasia, increased medial tone, and adventitial fibrosis as seen in **Figure 2**.[10]

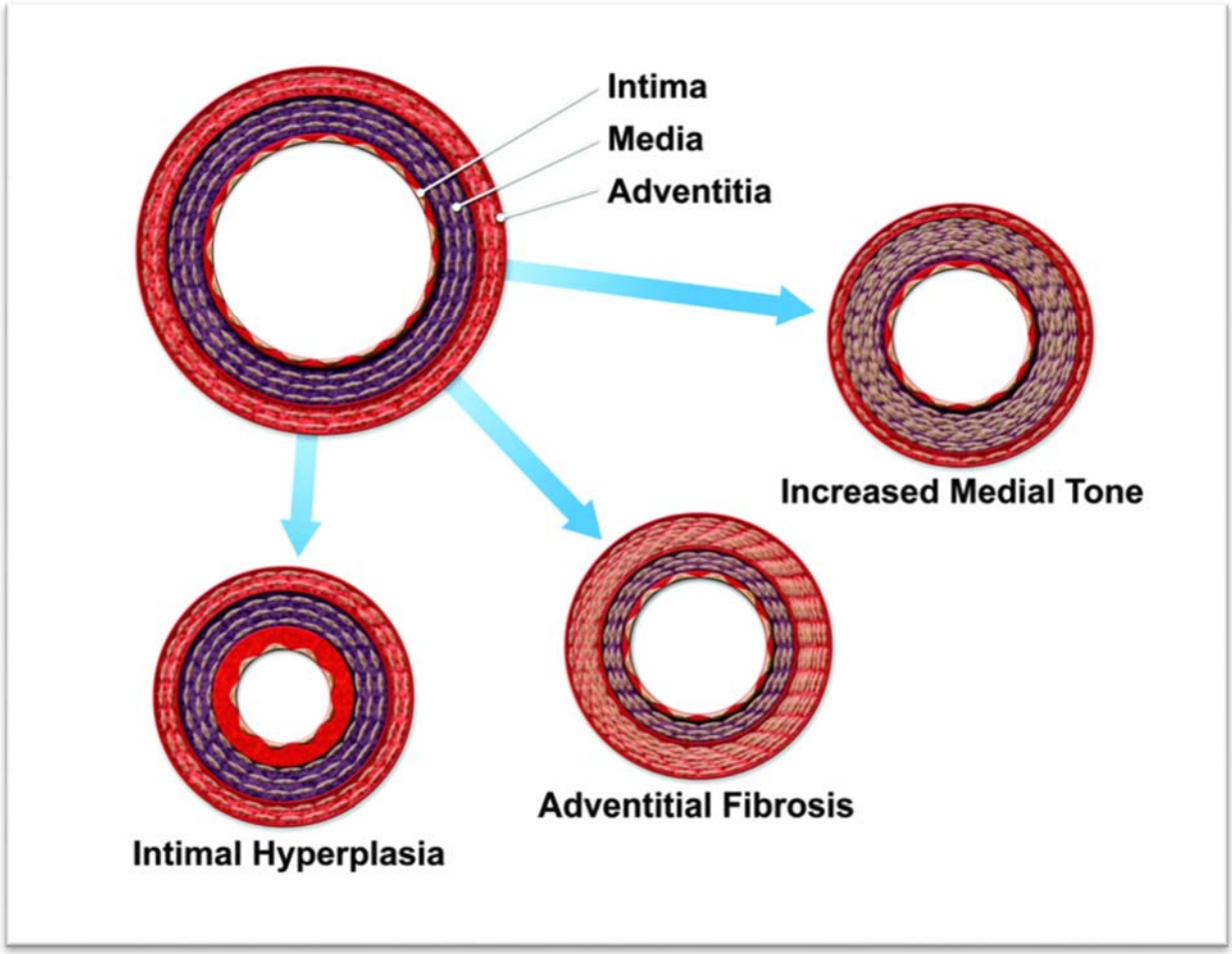

Figure 2: Vascular Remodeling in Arteries.[10] All three zones can become thickened due to vascular remodeling as seen in the intimal hyperplasia (as the deep red section becomes thickened), the increased medial tone (as the purple section becomes thickened) and the adventitial fibrosis (as the bright red/orange section becomes thickened). (Permission obtained from publisher of citation for use of illustration).

While remodeling occurs in all three zones, the course of vascular remodeling can either be directionally outward or inward.[11] The endpoint result of remodeling can also differ, either being hypotrophic (the overall vessel wall thickness decreases), eutrophic (the overall vessel wall thickness stays the same), or hypertrophic (the overall vessel wall thickness increases).[11] In the presence of increased systolic hypertension and increased pulse pressure due to aging, an outward vascular remodeling process occurs with both an increased medial tone and adventitial fibrosis.

The resultant remodeling in this case is also hypertrophic, where the vessel over time has an increased overall wall thickness. This type of vascular remodeling differs from the inward eutrophic remodeling characterized in classical essential hypertension for which current drugs treat.[11] There is no therapeutic available to stop outward hypertrophic vascular remodeling that can ultimately lead all the way to heart failure. Therefore, different molecular targets imperative to this process must be explored.

Tissue Transglutaminase Biochemistry

TG2 is a multi-functional enzyme that is shown to be ubiquitously produced in all vascular cell types.[5,12,13] The three main functions of TG2 include catalytic crosslinking, GDP/GTP binding, and fibronectin/collagen binding.[14] In the vasculature, the majority of TG2 has been shown to be intracellular yet catalytically inactive or in the closed GTP bound conformation as seen in **Figure 3A-B**. [12,14,19] When GTP is bound to the enzyme, the active site and active cysteine residue (Cys-277) is hidden.[12] Closed conformation TG2 also exists on the cell surface, where it can mediate different binding interactions with ECM proteins such as collagen and fibronectin along with integrin. [14] Open conformation TG2 exists in the presence of calcium ions (Ca^{2+}) and is mainly secreted into the ECM. [12,14,19,22] In the open conformation, the main TG2 catalytic crosslinking activity can either be inactive or active. In oxidative conditions, disulfide bonds between cysteine residues will block the active Cys-277 residue.[12] In reductive conditions, those disulfide bonds will be reduced and the active Cys-277 is available for catalytic crosslinking activity.[12]

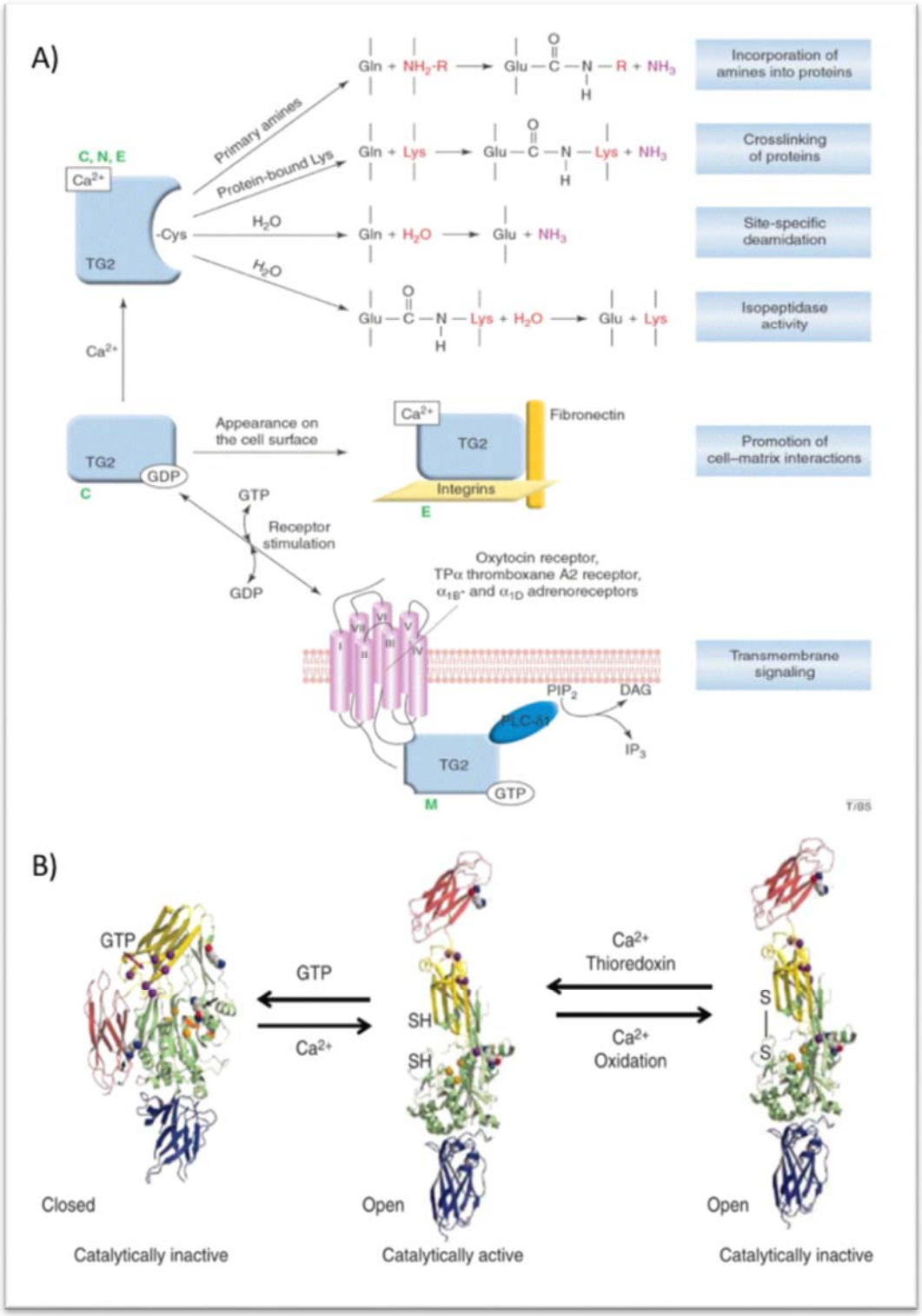

Figure 3: **Conformations and Functions of TG2**.[12,14] A) The different functions of TG2 based on localization are shown here. B) The three-distinct conformations based on GTP binding and Ca^{2+} concentration are displayed here. (Permission obtained from publisher of citations for use of illustrations).

TG2 specifically catalyzes the crosslinking of the glutamine side chain to primary amines. When the amine donor is a lysine side chain, TG2 creates an isopeptide bond that is stable and resistant to proteolytic cleavage as shown in **Figure 3A**.[14] By catalyzing this reaction between glutamine and lysine residues on ECM proteins, TG2 is able to establish a stable and highly organized ECM. In previous studies, we have shown that TG2 is the predominant transglutaminase involved in age related vascular/arterial stiffness, where ECM crosslinking promotes matrix deposition/accumulation and thus, increases vessel stiffness.[7] We have also previously shown that both expression and activity of extracellular TG2 are increased as the vasculature ages.[6]

Numerous changes occur in the vascular microenvironment with aging and hypertension including loss of NO bioavailability. We have previously shown that in the healthy vessel, normal levels of NO S-nitrosylate TG2 and constrain it to the intracellular compartment, thus effectively suppressing its crosslinking function.[6] In aging and hypertension, loss of NO correlates to reduced/ complete loss of TG2 S-nitrosylation, its secretion to and accumulation in the vascular ECM, and a striking increase in matrix crosslinking by TG2.[6]

Biomechanical Regulation of Proteins in Arteries

In human arteries, various mechanical forces are exerted on the different vascular cells types. Those include both a shear stress and a normal stress that lead to circumferential stretching of cells in the different zones of the artery, as seen below in **Figure 4**.

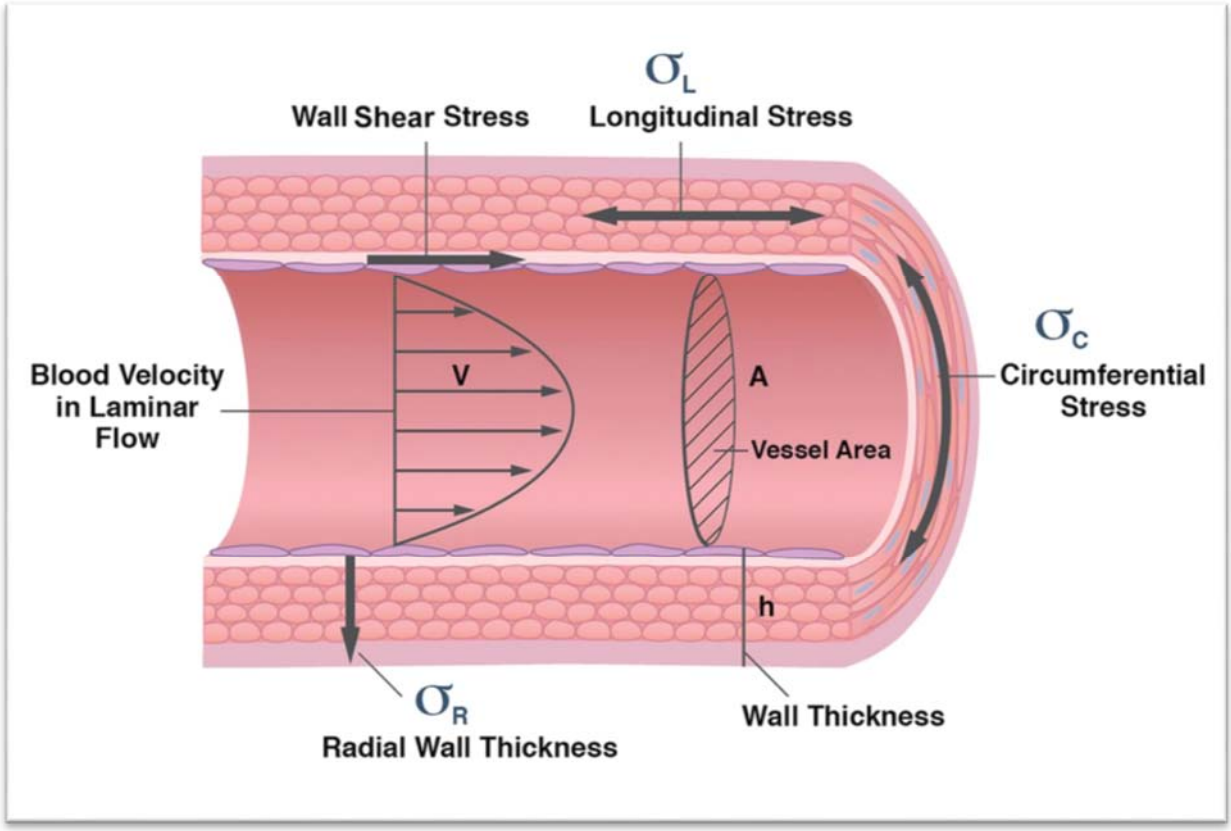

Figure 4: Biomechanical Stimuli on Arteries.[25] In the arteries, there is a wall shear stress exerted on the endothelial cell layer, a longitudinal stress and circumferential stress exerted on the endothelial and smooth muscle cells in the tunica intima and tunica media. (Permission obtained from publisher of citation for use of illustration).

These occur because in each cardiac cycle, the aorta expands during systole to accommodate the blood ejected into circulation and to absorb the resultant pressure pulse, and recoils during diastole. The pulsatile cyclic stretch exerted on the endothelial cells, smooth muscle cells, and fibroblasts occur at a stretching and relaxing frequency of around 1 Hz, assuming that the average heart rate is 60-70 beats per minute in a normal adult. The overall strain imposed on the cells by the hoop stress is dependent on the pulse pressure of the individual. Pulse pressure is defined as the difference between the systolic (ventricle contracting) and diastolic (ventricle filling) blood pressures. Chronic increases in pulse pressure due to elevated systolic blood pressure

imposes larger than normal cyclic strain on vascular cells. Cells can sense and respond to these mechanical forces in the microenvironment. Previously, it has been shown that the key arterial cells including fibroblasts, smooth muscle cells, and endothelial cells alter production of various structural and signaling molecules as a result of the mechanical stimuli resulting from cyclic stretching.[15-18] Overall, it is well-accepted that chronic exposure to high pulse pressure stimulates pro-fibrotic, matrix depositing pathways, leading to vascular remodeling. Increased wall thickness and renormalization of wall stress occur to prevent structural failure of the vessels. Here, we propose that TG2, a protein whose primary function is to deposit stable matrices, is activated by biomechanical forces in the vascular wall. TG2 regulation due to mechanical stimuli in vascular cells has not been studied or characterized, and thus, we wish to explore those aspects of TG2 regulation in this study.

Specific Aims

The overall goal of this project was to characterize the biomechanical regulation and activation of TG2 in large arteries leading to and maintaining arterial stiffening. Arterial stiffening and isolated systolic hypertension occur in a positive feedback loop with hypertension promoting continued arterial fibrosis and vice versa. Cells within large arteries participate in biomechanical sensing, which changes the expression of different proteins. Because of the nuanced bidirectional communication between cells and the matrix they reside in, the biomechanical stimuli sensed by these cells change because of arterial stiffening, and it is possible biomechanical regulation of TG2 expression plays an integral role in vascular remodeling. Another important aspect of TG2 biomechanical regulation and vascular remodeling is what aspect of TG2 function (i.e., crosslinking dependent vs crosslinking independent) contributes most to hypertension induced

arterial remodeling. To test this, we have created a mouse model expressing TG2 C277S catalytically inactive form of TG2.

The main aims/objectives of this project are to:

1) Characterize the biomechanical regulation of TG2 expression and secretion *in-vitro* in HASMCs and HAECs.
2) Characterize the biomechanical activation of TG2's crosslinking function *in-vitro* in HASMCs.
3) Determine the contribution of TG2's cross-linking function on loss of large artery compliance due to hypertensive remodeling.

EXPERIMENTAL DESIGN

Experimental Animals

TG2 -/- mice on a Black 6/129S mixed background, TGM2 C277S Heterozygous (HET) mice on a Black 6 background, TGM2 C277S Homozygous (HOM) mice on a Black 6 background, and WT Black 6 littermate mice (to HETs) were used in this study. TG2 -/- mice produce no TG2 protein, while the TGM2 C277S mice were created using CRISPR-Cas9 gene editing of the TGM2 gene to introduce a C→S mutation at the active site cysteine 277 that abolishes the TG2 cross-linking activity on one (HET) or both (HOM) alleles, while still producing a properly folded molecule recognized through western blotting. Animals were bred in-house, genotyped and sequenced accordingly to confirm each animal model. All animals were kept on the same food, water, and light exposure cycle. Additionally, all procedures performed on these animals were approved by and compliant with the regulations delineated by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Cell Culture

For this study, we used a variety of vascular cell types including human aortic endothelial and smooth muscle cell primary cell lines. These cell types were cultured using the following supplemented media: Human Aortic Smooth Muscle Cells (HASMCs) (ATCC) were cultured with complete smooth muscle cell media with 2% FBS, 1% (v/v) penicillin/streptomycin, and Smooth Muscle Cell Growth Supplement (ScienCell). Human Aortic Endothelial Cells (HAECs) were

cultured using complete endothelial cell media with 5% FBS, 1% (v/v) penicillin/streptomycin, and Endothelial Cell Growth Supplement (ScienCell).

Cell Stretching

Cells were subjected to uniaxial cyclic mechanical stimulation using the Strex Cell Stretching System to study the impact of biomechanical strain on Cell morphology and TG2 expression/secretion/function. Strex chambers were coated with human fibronectin (100 μg/mL) for 30 minutes at 37°C and 5% CO_2. The chambers were then washed with 1X PBS three times. HASMCs were seeded at a density of 150,000 cells per chamber, while HAECs were seeded at a density of 680,000 cells per chamber. After adhesion and growth overnight, the cells were serum starved with Insulin-Transferrin-Selenium (ITS) media (DMEM, 1X ITS, P/S) used for HASMCs and low serum (0.5% FBS) ECM media used for HAECs for 24 hours. Serum starvation media was replaced with fresh ITS or low serum ECM media respectively. The chambers were placed into the Strex Cell stretching device and were stretched in a pulsatile manner for 18 hours, set at a 1 Hz frequency, according to the strain variation described below (0%, 10%, 20%) in 37°C and 5% CO_2 conditions. Orientation of the cells was determined with 10x bright-field microscope images. The media and cell pellet were then collected for analysis with western blotting.

In Situ TG2 Cross-linking Activity Assay

The incorporation of FITC-conjugated cadaverine, a primary amine and well-known substrate of TG2, was determined by fluorescence microscopy to investigate TG2 activity in live

cells. Cells were seeded in STREX-mini chambers compatible with IHC procedures. After cell seeding and serum starvation for 24 hours, FITC-Cadaverine (100 μM, Thermo Fisher Scientific) in phenol-red free DMEM media supplemented with ITS was added for the duration of the stretching period (18 hours). After incubation, the cells were washed three times with PBS to remove excess FITC-cadaverine and fixed with 3.7% formaldehyde for 30 minutes. The cells were then blocked with 1% BSA in PBS for 1 hour. Following that, the cells were incubated with mouse monoclonal TG2 primary antibody (1:100) followed by DyLight 647 Conjugated Anti-mouse secondary antibody (1:200) with appropriate washes in between to label extracellular TG2. The nuclei were then stained with DAPI, and the cells were mounted appropriately. Cells were imaged at 10x, 20x, and 40x using a Nikon Eclipse 80i fluorescent microscope. The TG2 inhibitor T101 (10 μM) was used as a negative control for FITC-Cadaverine incorporation signal.

Western Blotting

Abundances of TG2 in the conditioned cell culture media, cell-derived matrix, and cytosol were determined using western blotting. GAPDH was used as a loading control where appropriate. After equal amounts of protein in all samples were ran through a gel, and transferred appropriately onto a nitrocellulose membrane, Ponceau-S staining was used to assess total protein levels on the blot. After washing and blocking with 3% Milk in TBST for 1 hour, the blot was incubated with TG2 Primary Antibody (1:1000 dilution) for 1 hour. After 3 washes with 1X TBST, the blot was then incubated with the appropriate mouse or rabbit HRP conjugated secondary antibody (1:5000 dilution) for 1 hour. Blots were imaged with Chemiluminescent ECL substrate using a Bio Rad

ChemiDoc system. Densitometry analysis was performed on all western blots, where Ponceau-S staining for total lane protein or GAPDH was used for normalization.

Open Conformation TG2 Quantification Assay

Abundances of open conformation TG2 in the conditioned cell culture media, cell-derived matrix, and cytosol were determined using western blotting after cells were incubated with Biotin-Ahx-MA-QPL-OMe (B015, 50 μM, Zedira). GAPDH was used as a loading control where appropriate. After equal amounts of protein in all samples were ran through a gel, and transferred appropriately onto a nitrocellulose membrane, a Ponceau-S stain was used to assess total protein levels on the blot. After washing and blocking with 3% BSA in TBST for 1 hour, the blot was incubated with Streptavidin HRP Secondary Antibody (1:1000 dilution) for 1 hour. After 3 washes with 1X TBST, the blots were imaged with Chemiluminescent ECL substrate using a Bio Rad ChemiDoc system. Densitometry analysis was performed on all western blots, where Ponceau-S staining for total lane protein or GAPDH was used for normalization.

TG2 Activity Assays: We examined TG2 activity in homogenized tissue to determine presence or absence of TG2 function in the various mouse models. TG2 Activity was assessed using two different crosslinking assays as described below:

Fluorescent Polarization Activity Assay

In this assay, we used the fluorescence polarization resulting from the crosslinking of FITC-Cadaverine with N, N-Dimethylcasein due to catalysis by TG2. Liver lysates were

obtained from samples from all four of the different animal models. After protein quantification, 50 μg of protein of protein was loaded into a 96-well black bottom dish in a total volume of 100 μL (protein in RIPA solution). The Complete N, N-Dimethylcasein solution was then prepared in Tris-HCl buffer (pH 8.0, 100 nmol/L) with N, N-Dimethylcasein at a concentration of 30 mg/mL, $CaCl_2$ at a concentration of 5 mmol/L, DTT at a concentration of 10 mM, and FITC-Cadaverine at a concentration of 200 nmol/L. 100 μL of the Complete N, N-Dimethylcasein solution was added to each protein sample and mixed thoroughly. RIPA control samples were also prepared to deduce the effects of RIPA for the baseline measurement. Lastly, each sample was run in triplicate with both no TG2 crosslinking inhibitor, and T101 (10 μM). After set up of the plate, the plate was incubated at 37°C and 5% CO_2 overnight. Using the FlexStation 3, a fluorescent polarization measurement in RFU's was obtained using medium sensitivity, a G factor of 1, and 485 excitation and 515 emission cutoffs.

BPA Incorporation Assay

Biotinylated pentylamine (BPA) is another amine donor that serves as a small-molecule substrate of TG2. TG2 activity in-situ or ex-vivo was assessed using a BPA assay. EZ Link Pentylamine-Biotin (Thermo Fisher Scientific) was incubated with intact liver samples at 37°C and 5% CO2 for a four-hour period. After that period, three 1X PBS washes were performed and the sample was homogenized to obtain a liver lysate. Protein quantification was performed on the lysate, and the samples were used for a dot blot assay. The blot obtained was blocked with 1% BSA in TBS for 1 hour and incubated with

Streptavidin HRP (1:1000 dilution) at 4°C overnight. The blot was imaged with Chemiluminescent substrate using the Bio Rad ChemiDoc system. Densitometry analysis was performed on the blot and normalized with a Ponceau-S stain.

Tensile Testing of the Aorta

The importance of TG2's main crosslinking function with age on elastic properties of the vasculature was further deduced using tensile testing on wild type, TG2 C277S HET, and TG2 knockout mice. Aortas were isolated from all three mouse lines, all from ages 10-12 months. They were cleaned of any excess fat and placed in Krebs Buffer that contained [in mmol/L] 118.3 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, and 11.1 dextrose with a pH of 7.4. The aortas were then cut into 1-2 mm rings for imaging. Bright field microscope images were taken (10x, length and lumen images) for both the intact rings. On a puller device (DMT), the pins were calibrated and positioned appropriately. Using the DMT 560 software and this calibrated puller device, the aortic rings were pulled apart with a displacement velocity of 50 μm/s until material failure. Using analysis described previously in Steppan et. al, the stress-strain curve coefficients were obtained to generate representative stress-strain curves.[8,26]

Pressure Myography

Pressure myography was used to assess the compliance, stretch, and strain of arteries from the different animal models described above both in the absence and presence of calcium, before and after remodeling in hypertensive conditions. The carotid arteries were dissected and cleaned

of any excess fat. They were then mounted onto steel cannulas in a chamber of the DMT Culture Myograph System. Each chamber was filled with Ca^{2+} free Krebs buffer (containing [in mmol/L] 118.3 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, and 11.1 dextrose at a pH of 7.4) and heated to 37°C. Using a peristaltic pump, fresh Ca^{2+} free Krebs buffer oxygenated with 95% O_2 and 5% CO_2 was used to continuously change the buffer within the chamber. With the DMT IR imager, the vessel walls were imaged with the pressure being regulated and set to specific values from 50 mmHg to 120 mmHg. Using the DMT Myoview II software, measurements of left wall thickness, right wall thickness, average wall thickness, inner diameter, and outer diameter were obtained. This was performed in the passive state (no Ca^{2+}) and in the active state (2.5 mM $CaCl_2$). After performing pressure-dimension analysis once, phenylephrine at 10^{-5} M was introduced to induce remodeling in Ca^{2+}- containing Krebs buffer and the sample was maintained at 100 mm Hg (hypertensive MAP) as a remodeling stimulus. After a 4-hour period, the initial passive and active dimension analysis procedure was repeated. To calculate vascular compliance, the following relationship was used:

$$Compliance = \frac{\Delta V}{\Delta P} \sim \frac{\Delta D_i^2}{\Delta P}$$

$$For\ a\ cylindrical\ sample$$

Statistical Analysis

All data are displayed as arithmetic mean ± standard error of the mean (SEM). Appropriate non-parametric tests were used to compare expression values (Kruskal-Wallis Test) since normality could not be assumed. Statistical significance was set at $P<0.05$ for all tests performed, and the appropriate levels of statistical significance and number of measures (n) are reported.

RESULTS

Pulsatile Stretching Regulates TG2 Secretion in HASMC

We first investigated the biomechanical regulation of TG2 due to pulsatile cyclic stretch in HASMCs. To do this, we utilized the Strex Cell Stretching System, in which HASMCs were seeded onto PDMS chambers and mechanically stretched at a controlled frequency and strain. To mimic the cell stretching imposed on vascular smooth muscle cells, we stretched the cells with a 1 Hz frequency for 18 h and varied the strain at 0% (no mechanical stimulation), 10% (normal physiological strain in the vessel wall in elastic arteries), and 20% (higher strain associated with increased pulse pressure in aging/hypertension). We see that with increasing magnitude of cyclic stretching, the HASMC morphology becomes elongated and there is alignment perpendicular with the direction of the stretch, thus indicating successful cell stretching. (**Figure 5A**) The media, cell pellet, and cell lysate were collected and processed. After western blotting for TG2, we see that there is a significant increase in total TG2 secretion to the ECM with increased strain ($P<0.0001$: **Figure 5B**).

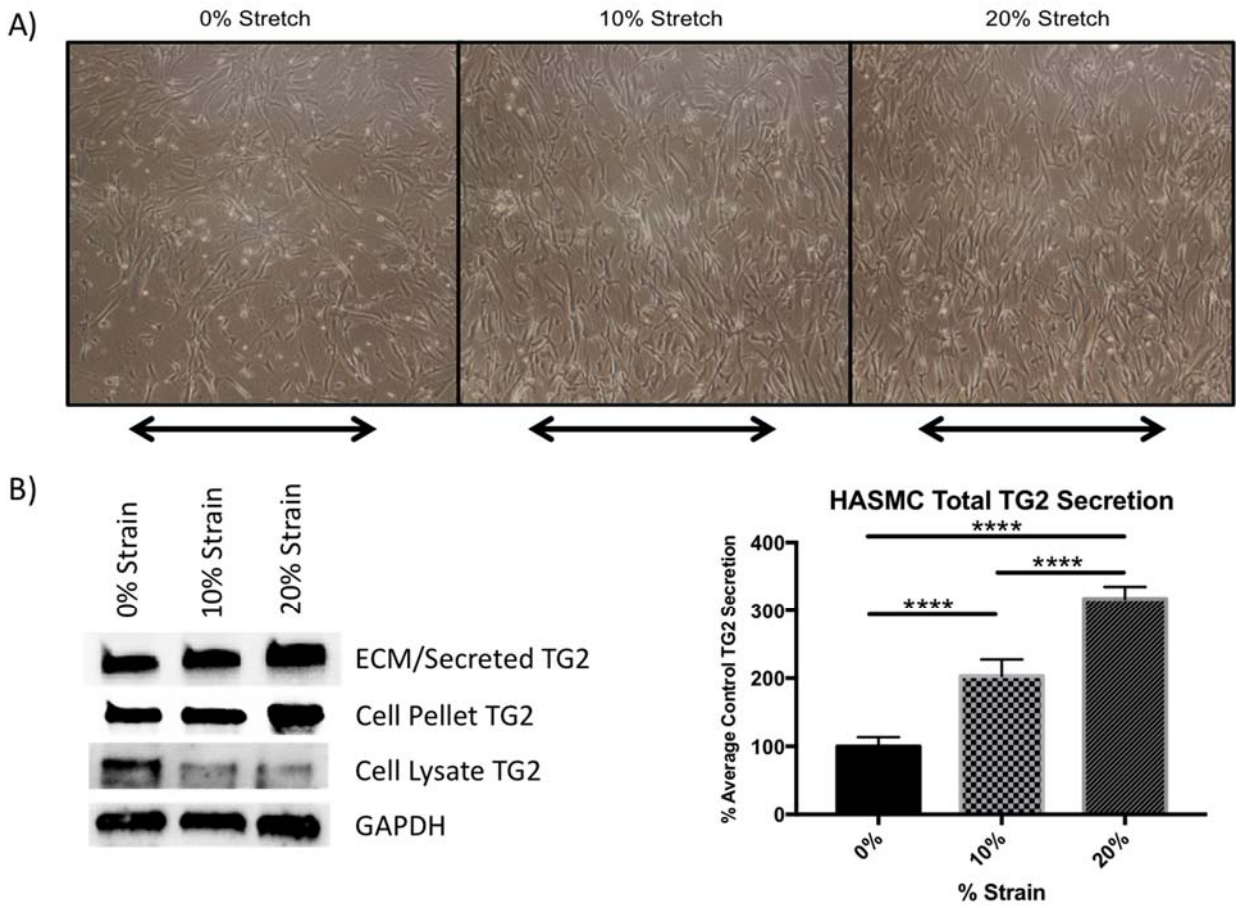

Figure 5: Cyclic Stretch of HASMCs Upregulates TG2 Secretion to the ECM. A) Bright-field images of the HASMCs after stretching (10x magnification). The perpendicular alignment and cell elongation increases as strain increases. B) Media western blots for TG2 Secretion to the ECM, TG2 in the Cell Pellet, and TG2 in the Cell Lysate. GAPDH loading Control shown also. TG2 secretion increases linearly with increasing strain (N=7, ****P<0.0001 using Kruskal-Wallis Test).

Next, HASMCs were subjected to the same cyclic stretching at various strains in the presence of Biotin-Ahx-MA-QPL-OMe (B015, 50 μM, Zedira), an irreversible inhibitor of TG2 that targets the crosslinking Cys-277 active site when TG2 is in its active and open conformation (**Figure 6A**). This will tell us how much active TG2 is secreted to the ECM. We again saw that the total TG2 secretion increases with increasing strain of the cyclic stretch (**Figure 5B**). However,

at 10% strain, the ratio of active, open-conformation TG2 to total TG2 decreases, and at 20% strain that ratio increases to a value larger than the static control (**Figure 6B**).

After looking at extracellular abundance and conformation of TG2 due to cyclic stretch in HASMCs, we next looked at the activity of TG2 cross-linking due to cyclic stretch. During the HASMC stretch time period, phenol-red free ITS media with FITC Cadaverine (100 μM, Thermo Fisher Scientific) was incubated with the cells. FITC Cadaverine is a fluorescently labeled molecule that can be catalytically cross-linked to the ECM by TG2. As the strain increases, we see that the nuclei orient perpendicular to the stretch, and the increased activity of TG2 seen in the increased FITC-Cadaverine incorporation (**Figure 6C**). TG2 was also stained for and as the strain increases, we also see an increase in TG2 present in the cell ECM (**Figure 6C**).

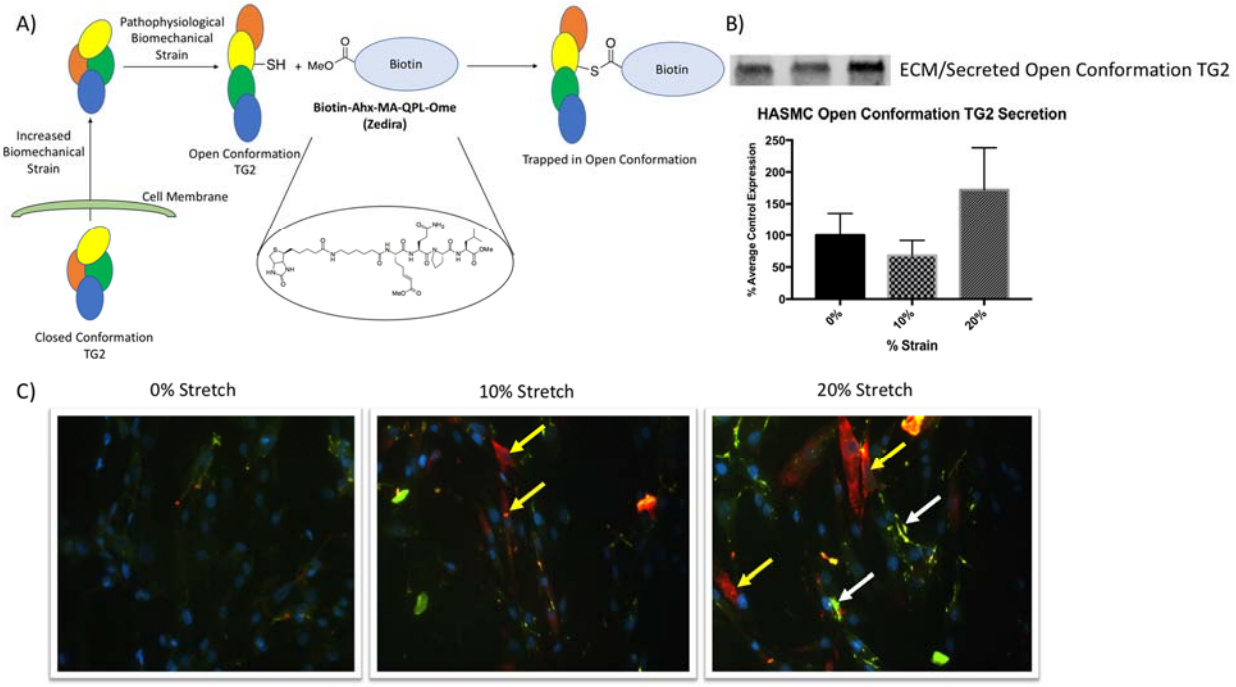

Figure 6: Cyclic Stretch of HASMCs Activates TG2. A) Flow diagram of use of Zedira irreversible inhibitor to test biomechanical strain induced secretion to the ECM and shift to the open, active conformation. B) Western blot of open, active conformation TG2 using the Zedira irreversible inhibitor. C) FITC incorporation assay images. Nuclei stained with DAPI (blue), FITC-Cadaverine incorporation displaying TG2 activity (green), and TG2 expression displayed (red). As strain of stretch increases, the TG2 expression in the ECM increases (yellow arrows), and the cross-linking activity increases as seen by FITC-Cadaverine incorporation (white arrows).

Pulsatile Stretching Regulates TG2 Secretion in HAEC

Next, we extended our study of biomechanical regulation of TG2 to HAECs, another relevant vascular cell type. The cell stretching protocol was identical to that of HASMCs, with the exception the cell seeding density that differed due to the size and morphology of HAECs. Here, after cell stretching, we again see an alignment profile perpendicular to the stretch vector and as the magnitude of stretch increases (**Figure 7A**). The endothelial morphology also becomes more elongated compared to the static control as the stretching strain increases, indicating a successful stretching of the cells (**Figure 7A**). The media and cell pellet/lysate were collected and processed, and a western blot for TG2 was performed. We see that the secretion of TG2 to the ECM peaks at the physiological (10% strain) and decreases at 20% strain but still remains higher than the static samples ($P<0.01$: **Figure 7B**).

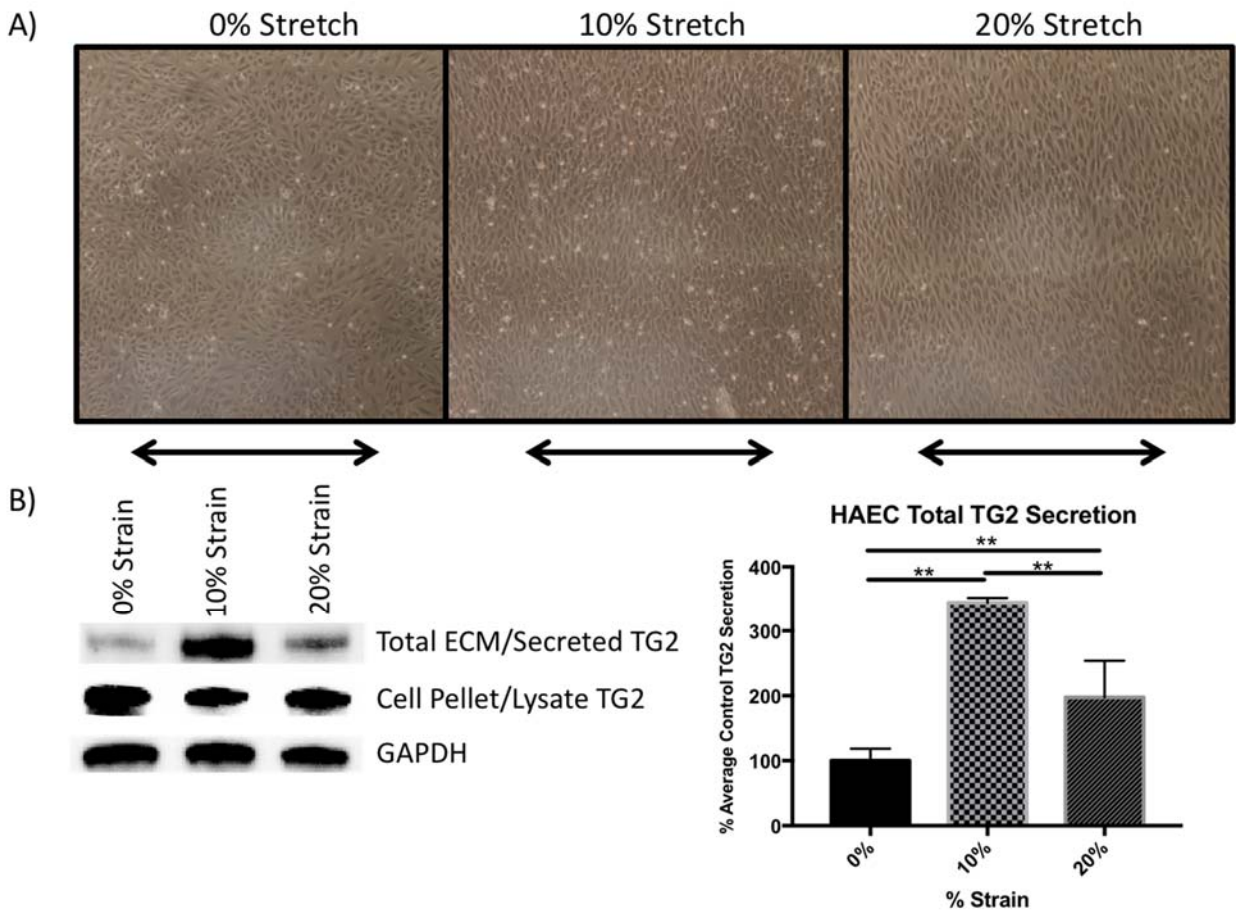

Figure 7: Cyclic Stretch of HAECs Induces Peak TG2 Secretion at Physiological Strain. A) Bright-field image (10x magnification) shows HAEC alignment perpendicular to the axis of stretch as the strain increases. B) HAEC TG2 secretions peaks at 10% strain and decreases at 20% (N = 5, **P<0.01)

TG2's Crosslinking Function Contributes to Aortic Modulus and Loss of Compliance

Recently, our lab has developed a novel TG2 C277S mutant mouse model by CRISPR-Cas9 mediated editing of the TGM2 gene, that has been by appropriate genotyping and sequencing. In this C277S mutant mouse, the active site Cys-277 residue is successfully replaced with a serine residue, thus rendering the TG2 molecule catalytically inactive. We successfully generated mice that were HET (1 mutant allele, 1 WT allele), HOM (both alleles bear mutation), and WT littermate

controls. We first needed to confirm our mouse model on an *in-vivo* protein level. To do this, we isolated liver tissue from TG2 C277S HET, TG2 C277S HOM, and littermate WT mice. TG2 -/- mice were used as controls to represent complete loss of TG2 protein. The tissue was crushed and homogenized appropriately and the lysate was used to examine TG2 expression (western blot) and TG2 cross-linking activity (FP assay). The western blot showed expression of TG2 in the TG2 C277S HET, TG2 C277S HOM, and WT mice, but not in TG2 -/- mice (**Figure 8B**). The FP TG2 activity assay showed decreased activity to the TG2 C277S HET mouse, and negligible activity in the TG2 C277S HOM and TG2 -/- mice as expected (**Figure 8A**). These findings confirm that the mutation successfully targeted the intended function of TG2.

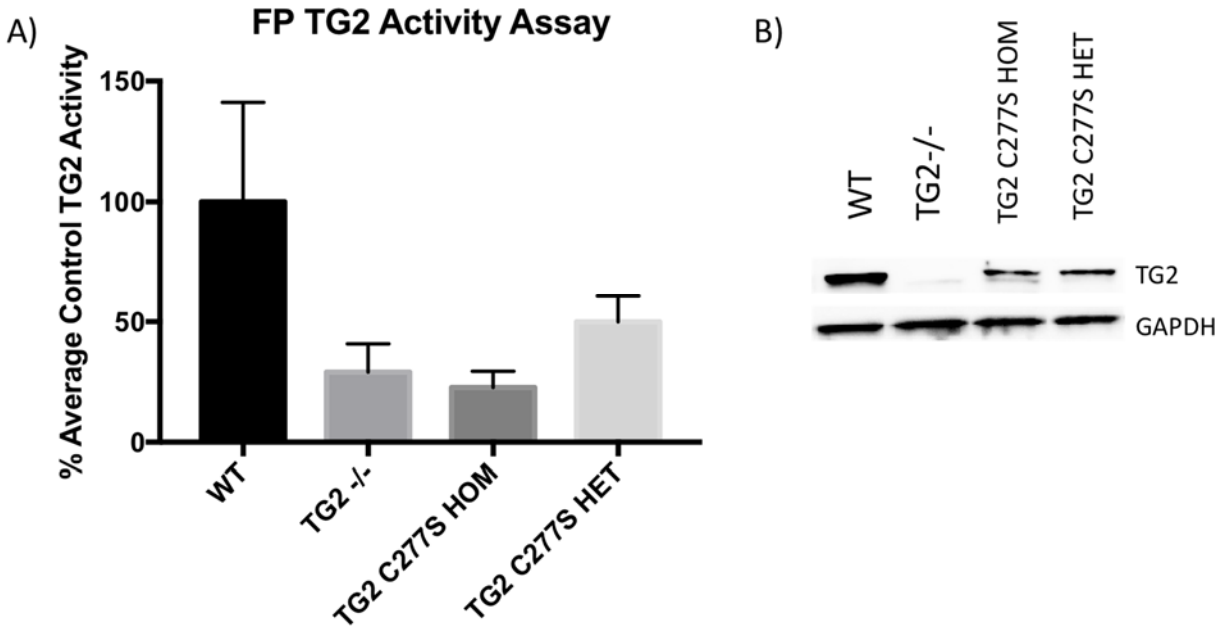

Figure 8: TG2 C277S CRISPR Mouse Produces Catalytically Inactive TG2. A) FP Assay TG2 specific activity given as the percent average control. B) Western Blot showing TG2 expression in all liver lysates except the TG2 -/- sample, and GAPDH as a loading control.

After successful confirmation of the reduced activity associated with the TG2 C277S HET and HOM mice models, the compliance of the vessels was assessed using pressure myography. Starting with passive compliance analysis of the common carotid artery (no muscular contraction),

we see that the pre-remodeling compliance plotted as D_i^2 versus pressure, the compliance varies as WT < TG2C 277S HET < TG2 C277S HOM, TG2-/- (**Figure 9A**). After a four-hour period of phenylephrine induced remodeling under constant hypertensive mean arterial pressure (110 mmHg), the passive compliance analysis was repeated for all samples. We see that while WT show a significant reduction in compliance (= increased stiffness), the TG2-/-, HOM, HET do not show any significant loss of compliance in remodeling. This is illustrated in the D_i^2 versus pressure plot (**Figure 9B**). Furthermore, the linear slope of each of the D_i^2 versus pressure plots for each sample were taken to obtain overall compliance of the samples in [μm^2/mmHg]. Comparing these values, we found that the mean compliance of the WT was less than that of the TG2 C777S HET, which was less than both the TG2 C277S HOM and TG2 -/- mice (**Figure 9C**). After hypertensive remodeling, we see a greater loss in compliance of the WT samples ($P<0.05$), with virtually no loss of compliance seen in any of the other animal models.

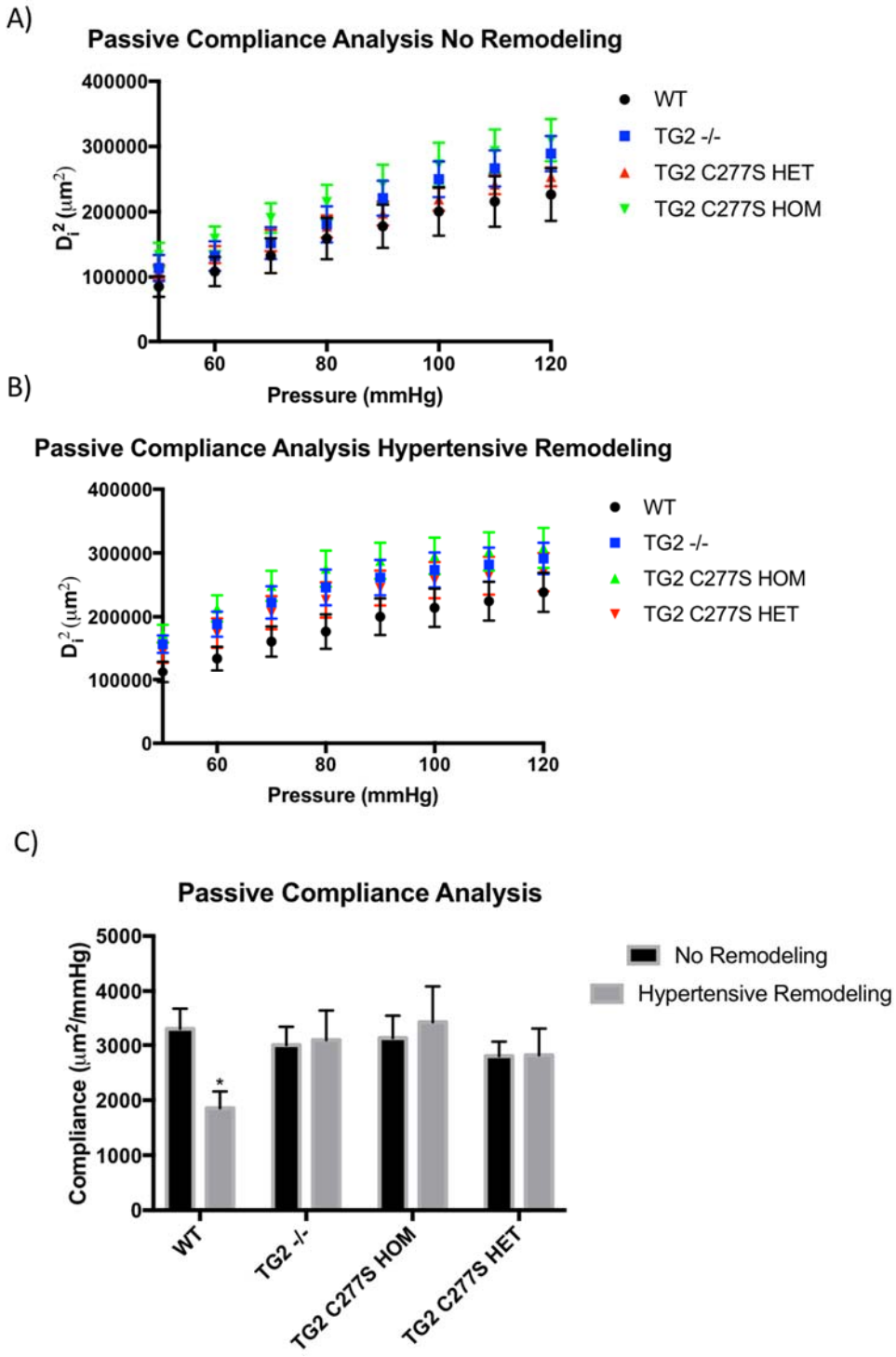

Figure 9: Passive Compliance Analysis After Hypertensive Remodeling. A) Prior to remodeling, passive compliance was plotted as D_i^2 versus pressure, showing the relationship between the initial properties of the carotid arteries in the mouse models. B) Passive compliance was plotted after the four-hour hypertensive remodeling period. C) The active compliance of each sample for each animal model before and after hypertensive remodeling was compared. (N=5 Animals for Each Condition, *P<0.05).

After testing passive compliance of the vessels both before and after hypertensive remodeling, tensile testing was performed on the intact aortas of the animal models as a measure of vessel stiffness. The stress vs. strain relationship (elastic modulus) was obtained from aortic rings of the WT, TG2 -/-, and TG2 C277S HET animal models in two different age groups (6-7 months, 10-12 months). In the 10-12 month old mice, we see a steeper curve in the WT than in the TG2 -/- and TG2 C277S HET (P<0.0001: **Figure 10A**). The TG2 -/- and TG2 C277S HET mice appear to be very similar. In the 6-7 month old mice, again we see a steeper curve in the WT mice than in the TG2 -/- and TG2 C277S HET (P<0.0001: **Figure 10B**). Also, again we see that the TG2 -/- and TG2 C277S HET curves appear to be very similar. There also is a striking difference in the curves looking at age as a factor. In the older mice plots, the stress needed for the corresponding strain is less than in the younger aged mice plots (i.e., samples from older mice are stiffer). This is true for all animal models.

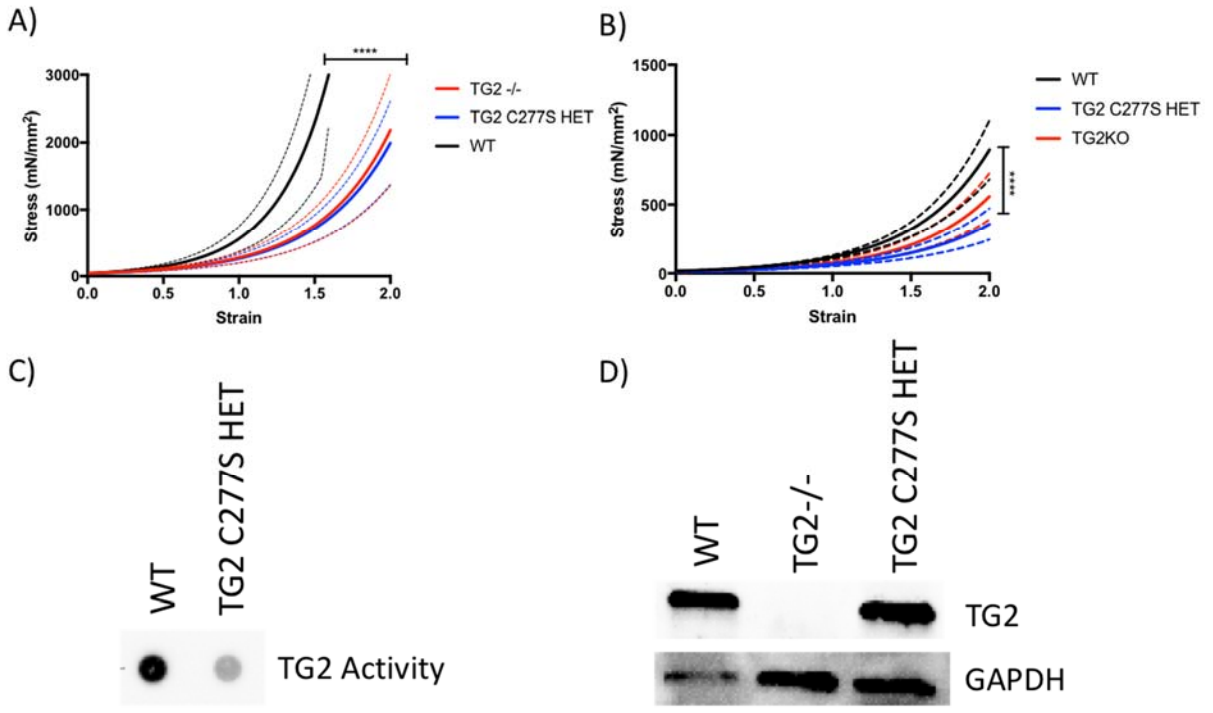

Figure 10: TG2 Crosslinking Contribution to Elastic Modulus. A) Stress vs. Strain (Elastic Modulus) for 10-12 month mice (N=6 Animals For Each Condition, ****P<0.0001). B) Stress vs.

Strain (Elastic Modulus) for 6-7 month mice (N=3 Animals For Each Condition, ****P<0.0001). C) BPA activity assay showing reduced TG2 activity in the TG2 C277S HET mouse than the WT in liver lysate samples. D) Western blot showing TG2 expression in aortic samples for WT and TG2 C277S HET mice, and no TG2 expression in the TG2 -/- mice. GAPDH used as loading control.

Overall vascular stiffness is the sum total of the contributions from the two-primary load-bearing elements: the vascular ECM and SMC. The passive compliance analysis on the common carotid artery and the tensile testing of the aorta help characterize the importance of TG2 crosslinking on relaxed arterial stiffness (i.e., absence of SMC tone). Next, active (contracted) arterial compliance analysis was performed in the same common carotid arteries for each animal type. Pre-remodeling, we see the same pattern where the WT plots are below that of the TG2 C277S HET, which is below the TG2 -/- and TG2 C277S HOM models (**Figure 11A**). After hypertensive remodeling, we again see a loss of compliance in the WT, but not in the TG2 -/-, TG2 C277S HET, and TG2 C277S HOM samples (**Figure 11B-C**).

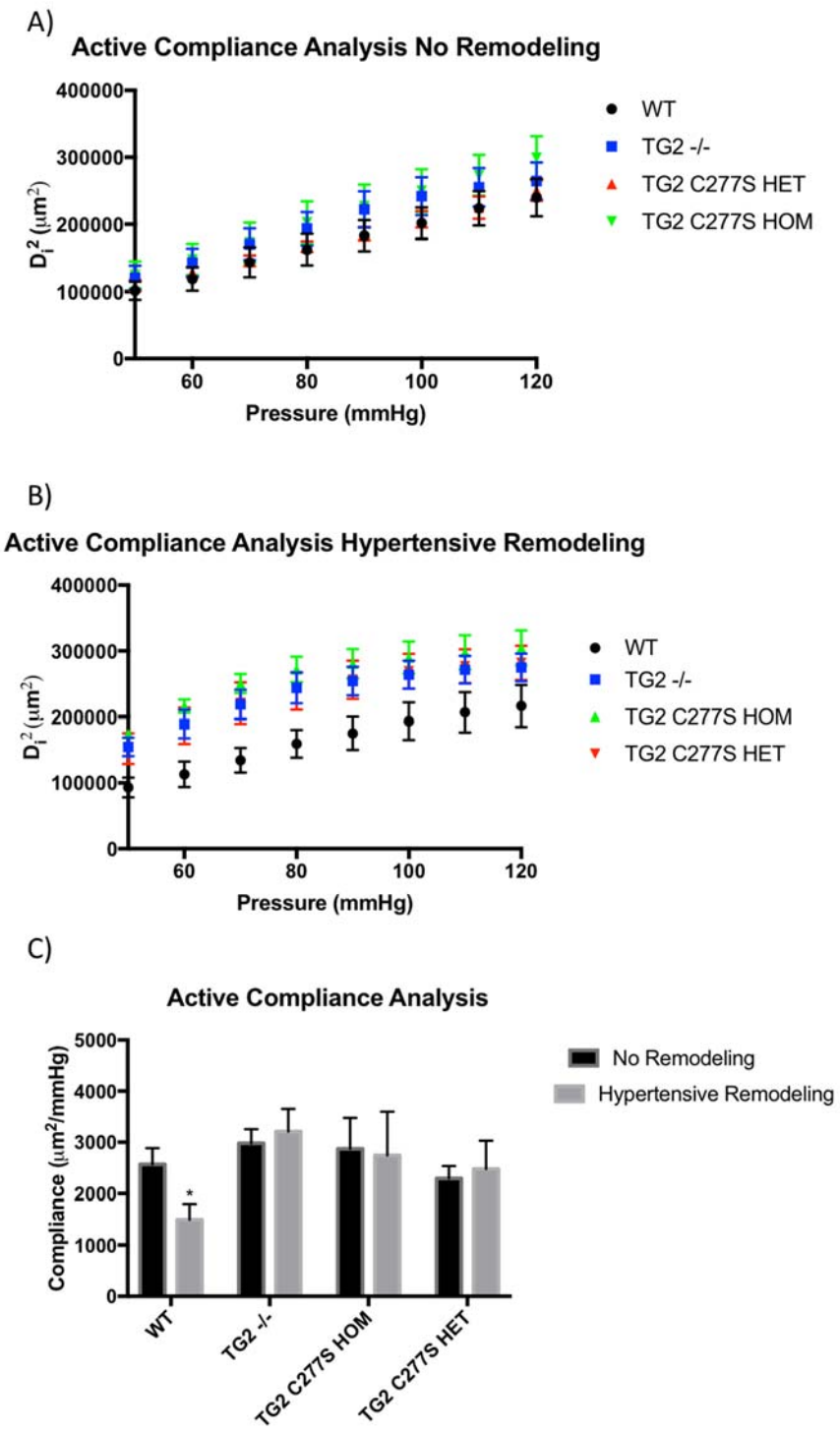

Figure 11: Active Compliance Analysis After Hypertensive Remodeling. A) Prior to remodeling, active compliance was plotted as D_i^2 versus pressure, showing the relationship between the initial properties of the carotid arteries in the mouse models. B) Active compliance was plotted after the four-hour hypertensive remodeling period. C) The active compliance of each sample for each animal model before and after hypertensive remodeling was compared. (N=5 Animals for Each Condition, *P<0.05).

DISCUSSION

In this study, we attempted to further characterize biomechanical TG2 regulation and elucidate the importance of TG2's crosslinking functionality in large artery remodeling. Our results indicate that cellular regulation of TG2 secretion to the ECM differ in HAECs and HASMCs, potentially indicative of a progressing outward vascular remodeling profile in large arteries in the presence of isolated systolic hypertensive conditions. Furthermore, TG2 crosslinking directly contributes to the aortic elastic modulus and is essential for vascular remodeling under hypertensive conditions.

Cellular Level Biomechanical Regulation of TG2 Suggestive of Outward Arterial Remodeling

As we age, arterial structure is modified through matrix crosslinking, in which TG2 is a major player.[6,20,23,26] Initially, in large arteries and the development of classical hypertension, intimal hyperplasia occurs leading to a thickened intima layer.[23] This stiffening leads to isolated systolic hypertension development and an increase in pulse pressure, which simultaneously increases hoop stress experienced by cells in the artery. This results in positive feedback loop wherein the augmented biomechanical stress promotes an outward remodeling profile, worsening the arterial stiffening. With advancing age, the stiffening and hypertension progress to a point that the risk of a serious adverse cardiac event becomes imminent.[1,2]

Smooth muscle cells in the vessel wall sense and respond to the increased magnitude of stretch by depositing additional matrix, by enlarging (hypertrophy), and dividing (hyperplasia). In terms of mechanics of the vessel, these cellular events facilitate the thickening of the vessel wall

to restore wall stress to levels similar to vascular homeostasis. We predicted that TG2 is a key enzyme activated to promote vessel wall remodeling in response to pathological biomechanical stress. In our cellular level regulation experiments, we studied how the change in the magnitude of biomechanical stimulation of cells within large arteries could affect the expression and activation of TG2 crosslinking and how it relates to the remodeling pattern occurring. In the vessel wall, endothelial and smooth muscle cells express high levels of TG2 at basal conditions. Thus, we focused our efforts on these two cells types.

In HAECs, we found an increase in TG2 secretion to the ECM from static conditions to exposure to cyclic stretch at 10% strain (normal physiological conditions). The expression of TG2 was highest at physiological stretch. We suggest that this peak in TG2 secretion from endothelial cells under normal physiological conditions compared to static conditions is consistent with the need of the tunica intima endothelial layer to be a strong and selective barrier. Surprisingly, and contrary to our expectation, we found a decrease in TG2 secretion to the extracellular space at pathophysiological conditions (20% strain). The loss of extracellular TG2 in HAEC at pathological stretch (when compared to physiologic stretch) could be due to EC responses to promote cell adhesion at this high strain by retaining TG2 at the cell surface rather than releasing it to the matrix, therefore leading to decreased cell proliferation, or reduced cell viability. The specific mechanistic cause for this observation remains to be verified in further studies.

The HASMCs, on the other hand, continue to increase TG2 secretion with increasing magnitude of strain in a linear fashion. Intriguingly, we observed that while TG2 abundance in the extracellular space increased with strain, crosslinking function was only markedly increased at pathological (20%) strain. We thus predicted that in the case of SMCs, mechanical forces facilitate a conformation change in TG2 from closed to open, unmasking its active site cysteine. We tested

this hypothesis using the irreversible TG2 inhibitor (Biotinylated-Ahx-MA-QPL-OMe) that binds to open conformation of TG2 and traps it in the open conformation. Indeed, the open conformation or catalytically active TG2 declines at physiological strain (10%) compared to the static control but increases at the pathophysiological strain from increased pulse pressure (20%), suggesting that pathological mechanical forces activate TG2 in the vascular media. Importantly, this finding is consistent with other studies that show TG2 to be the primary mediator of matrix deposition in the early phase of remodeling.[24]

Based on these findings, we suggest that as the pulse pressure begins to rise as we age, we see an upward shift in the expression and activation of TG2, prominently in the HASMCs and less so in the HAECs. This points towards a remodeling of the tunica media and elastic lamina as a function of pulse pressure increase. The mechanical regulation of TG2 secretion on the cellular level confirms the importance of TG2 in the outward hypertrophic remodeling associated with isolated systolic hypertension, providing evidence strongly in favor of TG2 being a therapeutic target for isolated systolic hypertension due to aging.

Importance of TG2 Crosslinking Function to Vessel Compliance and Stiffness

Next, in this study we characterized the importance of TG2 crosslinking to large artery stiffness, compliance, and arterial remodeling. After tensile testing of the intact aortas from the WT, TG2 -/-, and TG2 C277S HET mouse models, we see that WT vessels are remarkably stiffer than the TG2 -/- and TG2 C277S HET vessels. The TG2 -/- and TG2 C277S HET have relatively the same stiffness. Previously, our lab has shown a difference in aortic stiffness in WT and TG2 -/- mice, so that difference seen above is as expected.[7] The TG2 C277S HET mouse has one WT

TG2 allele and one C277S TG2 mutant allele. The activity was shown to be reduced, but not abolished as in the TG2 -/- mice, which is seen in the BPA and FP activity assays performed on the mouse models. We thus expected that the mechanical modulus of the HET mice would be intermediate to WT and TG2-/-. Surprisingly, however, the TG2 C277S HET aortic stiffness is relatively the same as the TG2 -/-, and is much less stiff than the WT, despite the presence of TG2 crosslinking function. Akker et. al determined that TG2 secretion outside of the cell most likely occurs through the production of a micro particle vesicle system, which eventually gets transported to the cellular membrane.[25] They found in their study that a minimum threshold of TG2 cross-linking activity is needed for the TG2 to be secreted from the cell membrane.[22] This would explain our results in this case of the TG2 C277S HET mutant mouse, as the activity is reduced by about a factor of 2, which might not be enough to meet the threshold for TG2 secretion to the ECM. This would explain the less stiff vessels we see in the results of our study. This remains to be confirmed in further studies.

After determining the decreased stiffness of the aortas of TG2 -/- and TG2 C277S HET mice, we next performed pressure myography to determine the compliance of common carotid arteries from the mouse models. Looking at the passive carotid artery compliance using pressure myography, we see that initially there is a modest difference in vessel compliance. The TG2 -/- and TG2 C277S HOM mice were more compliant than the TG2 C277S HET mice, which was more compliant than the WT mice. This is consistent with our findings that TG2 -/-, TG2 C277S HOM, and TG2 C277S HET are less stiff than WT aortas. However, the differences in this case are not as large as in the aorta, as there is a minimal difference in common carotid artery compliance initially. The key difference occurs after phenylephrine induced remodeling under hypertensive conditions (110 mmHg consistent pressure). There is a significant loss of compliance

in the WT vessels, with no loss of compliance in the TG2 -/-, TG2 C277S HOM, and TG2 C277S HET vessels. This suggests that TG2 crosslinking is solely responsible for the loss of compliance during this remodeling period. In the active compliance analysis, we see the exact same trend as in the passive compliance analysis. The only difference is that the loss of compliance in the WT vessels appears to be larger in the contracted state rather than the relaxed state of the vessel. The increased loss of compliance during contracted analysis is indicative of the potential addition of more smooth muscle cells. This suggests that TG2 crosslinking is necessary for smooth muscle cell migration. Overall, the large artery analysis of compliance and stiffness in these animal models proves that TG2 crosslinking is essential for isolated systolic hypertension induced remodeling. In this study, we used a 4 h remodeling period, which is sufficient to study the initial vessel response. Experiments in which the remodeling stimulus is maintained for longer duration (24-48 h) would further yield the relative contributions of TG2 to early vs. late remodeling mechanisms.

CONCLUSION

In this study, we demonstrated that TG2 abundance is intimately linked to biomechanical strain in both a cellular and physiological level. We further show that different cell types have distinct responses to increasing magnitudes of strain ranging from static to hypertensive, likely due to the distinct roles these cells play in vascular homeostasis and pathobiology. We also showed that TG2 crosslinking function plays a central role in basal aortic stiffness. We finally showed using novel mouse models that ablation of TG2 crosslinking function prevents biomechanical strain induced medial remodeling in vessels.

FUTURE WORK

This study yielded some surprising findings that can be further pursued to obtain mechanistic insights on TG2 regulation and vascular stiffening. Specifically, the HAEC show a peak secretion of TG2 at physiological stretch and a decline in TG2 secretion in pathological conditions. The specific mechanisms leading to this could include 1) retention of TG2 at the cell surface to promote EC adhesion, 2) transcriptional regulation of TG2 in ECs, 3) reduced cell proliferation in response to pathological strain, or 4) increased cell death/reduced viability in response to pathological strain. Moreover, ECs are also continuously exposed to shear stress due to the flow of blood. The combined effects of stretch and shear can further alter TG2 secretion by ECs.

Next, contrary to our expectation, the HET mice were more similar to the HOM and TG2-/- mice, rather than WT mice. The possible mechanisms include 1) a threshold TG2 activity is required to successfully secrete TG2 via exosomes, 2) a small loss in TG2 crosslinking function propagates as a high loss in stiffness – this is actually quite intriguing because this suggests that in aging and hypertension, a partial inhibition of TG2 may be adequate to preserve vascular mechanical properties, or 3) the in the HET mice, the WT allele is somehow silenced either during development or postnatally, thus expressing only the mutant version of TG2. This is further interesting as this suggests that the mutant TG2 proffers some benefits during growth. Using pulse wave velocity measurements, it will be possible to investigate the contribution of TG2 crosslinking function to in vivo stiffness. Finally, the specific differences between the HOM and HET mice can further be studied in natural aging over the course of the lifetime of the animal, which was outside the scope of this study.

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CURRICULUM VITAE

Sean Melucci

sean.melucci@gmail.com

Phone: (408) 348-5604

Education

Johns Hopkins University, Baltimore, MD June 2017-May 2018
Major: M.S.E. Chemical and Biomolecular Engineering, Essay-Based
Essay Title: "Biomechanical Forces Activate Tissue Transglutaminase Resulting in Vascular Remodeling and Stiffening"

Johns Hopkins University, Baltimore, MD August 2013-May 2017
Major: B.S. Chemical and Biomolecular Engineering, Molecular and Cellular Bioengineering Concentration
Minor: Psychology

Research Experiences

JHMI Anesthesiology and Critical Care Medicine: Dr. Santhanam Lab, Baltimore, MD June 2015-May 2018
Research Assistant
Biomechanical Regulation/Activation of TG2 and Roles of TG2 in Vascular Remodeling Project

- Produced both viral and non-viral clones of different mutant TG2 genes that produce the mutant forms of TG2 with specific functions inhibited to ascertain different roles of TG2 in vascular remodeling (published in the Journal of the American Heart Association on a paper regarding TG2 roles in vascular remodeling)
- Analyzed the roles of cell stretching and biomechanical forces on the regulation and activation of TG2 in the vasculature using uniaxial cell stretching expression and activity assay experiments
- Tested the characteristics of vessel stiffness and vessel compliance on TG2 -/- and CRISPR TG2 C277S mutant mouse models compared to WT mice using tensile testing and pressure myography

Bioreactor Project

- Created and optimized a filtration seeding technique for rat and mice aorta samples in order to produce re-cellularized aorta tissue scaffolds with an ideal and even cellular spatial distribution and density
- Developed a flow bioreactor system to obtain better de-cellularization of scaffolds to retain more structural integrity of the extracellular matrix

JHU Chemical Engineering Car, Baltimore, MD August 2014-June 2016
Team Member (Propulsion Mechanism)

- Optimized the Zinc-Air battery with a copper catalyst used for JHU's 2015 Regional Competition Winning/2015 Nationals Competitor Car
- Performed experiments testing the reliability of the batteries over time to ensure that the needed voltage and amperage was maintained
- Designed the schematic for the use of hydrogen fuel cells for JHU's 2016 Regional Competition Car with the necessary safety features

Project Lab: Phage Hunting, Baltimore, MD August 2013-May 2014
Class Member, Lab Researcher

- Isolated a unique bacteriophage specific to *Mycobacterium smegmatis* from a sample of dirt
- Cultivated the isolated bacteriophage into high concentrations in order to isolate and purify its DNA, sequence it, and annotate the genome using software such as DNA Master, Phamerator, and BLAST comparison results
- Performed an individual research project looking at the amplification of my unique bacteriophage when cultivated with bacteria in the presence of different metal cations

Work Experience

JHU Transport Phenomena in Practice Teaching Assistant, Baltimore, MD January 2018-May 2018

- Worked as a teaching assistant for the graduate level Transport Phenomena in Practice class in which I held recitation sections with practice problems and answered any questions that the students had
- Responsible for grading assignments/exams and utilized Blackboard to manage all of the course materials (assignments, class lectures, gradebook)

JHU Organic Chemistry Lab Teaching Assistant, Baltimore, MD August 2015-May 2017

- Worked as a laboratory teaching assistant assigned to 13 students with the responsibilities of demonstrating proper lab technique and supervising the students while grading their technique
- Taught students how to think ahead while working in a lab to be more efficient and helped students with any questions or concerns that they had about the course

Community Service Experience/Extracurricular Activities

Mercy Medical Center Emergency Department, Baltimore, MD May 2017-May 2018
Volunteer

- Worked directly under the charge nurse where I assisted with bringing patients back to rooms and interacting with them to help make their stay as comfortable as possible, which included getting them things like blankets, food, and water as allowed by the doctor
- Assisted and shadowed the charge nurse triage certain patients brought in by an ambulance as well as other various procedures
- Helped manage patient charts by making sure they were complete and available for the doctors and nurses when needed

Thread, Baltimore, MD September 2013-September 2016
Head of Family, Mentor, Team Member

- Led a group of mentors for a Thread Student who was chosen for this program based on failing high school grades and potential for improvement
- Responsible for fostering relationships between me and all the mentors in my group with the student by organizing Thread family events, tutoring sessions, and community service programs as well as continually creating a calendar of tasks that need to be completed
- Tellvon's (my Thread student) GPA and attendance improved dramatically throughout this experience so that he is passing and on track with his community service requirements

Thread Summer Research Impact Internship, Baltimore, MD June 2015-August 2015

- Taught a Thread High School Student proper etiquette while working in a lab from punctuality to efficient modes of communication
- Showed the student various laboratory techniques that are important and necessary to the production of results from the lab